PTO 1390 Page 1 of 1 | US Dept. of Commerce Pat. & Trademark Office | Attorney's Docket No. 21437

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

US. Application No. (if known) 09/529043

INTERNATIONAL APP. NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/06210	30 September 1998	4 October 1997

TITLE OF INVENTION

METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE SPARTATE AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METH

APPLICANT(S) FOR DO/EO/US

Bernd EIKMANNS et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EU/US) the following .

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3. ☐ This is an express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 317(b) and PCT Articles 22 and 39(1).
4. ☑ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☑ A copy of the International Application as filed (35 USC 371(c)(2)).
   a. ☑ is transmitted herewith (required only if not transmitted by the International Bureau.
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Patent Office.
6. ☑ A translation of the International application into English.
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau.
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3).
9. ☑ An oath or declaration of the inventor(s) (35 USC 371(c)(4).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

Items 11. to 16. below concern documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☑ An Assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☑ Other items of information.
    Drawing (2 sheets)
    References
    PTO-1449

US Application no (if known)	International Application no.	Attorney's Docket No.
09/529043	PCT/EP98/06210	21437

17. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
Basic National Fee (37 CFR 1.492(a)(1)-(5):		
Search report has been prepared by the EPO or JP $840.00		
Int'l prel. exam. fee paid to USPTO (37 CFR 1.482) $670.00		
No int'l prel. exam. fee paid to USPTO (37 CFR 1.482) but int'l search fee paid to USPTO (37 CFR 1.445(a)(2) $690.00		
Neither int'l prel. exam fee (37 CFR 1.482) nor int'l search fee (37 CFR 1.455(a)(2)) paid to USPTO $970.00		
Intl. prel. exam. fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Art. 33(2-4) $96.00	**$970**	
ENTER APPROPRIATE BASIC FEE AMOUNT		
Surcharge of $130.00 for furnishing oath or declaration later than □ 20 □ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

CLAIMS	NO. FILED	NO. EXTRA	RATE		
Total claims	**37**	**17**	$18	**$306**	
Ind. claims	**0**	**0**	**$78**	**$0**	
MULTIPLE DEP. CLAIM(S) (if applicable) **(see prel. amt.)**			**260**		
			TOTAL OF ABOVE CALCULATIONS	**$1,276**	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (37 CFR 1.2, 1.27, 1.28)				**$0**	
			SUBTOTAL	**$1,276**	
Processing fee of $130.00 for furnishing the English translation later than □ 20 □ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
			TOTAL NATIONAL FEE	**$1,276**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The Assignment may be accompanied by an appropriate PTO-1595 cover sheet (37 CFR 3.28, 3.39)				**$40**	
			TOTAL FEES ENCLOSED	**$1,316**	
				Amt to be refunded	
				Amt to be charged	

a. ☑ A check in the amount of **$1276** to cover the above fees is enclosed
☑ A check in the amount of **$40** to cover recordal of the Assignment
b. □ Please charge my deposit account 18-2025 **$00.00** to cover the above fees. A copy of this sheet is enclosed.
c: ☑ The commissioner is authorized to charge any additional fees which may be required or credit any overpayment to deposit account 18-2025. A copy of this sheet is enclosed

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:

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09/529043

528 Rec'd PCT/PTO 03 APR 2000

CERTIFICATE OF EXPRESS OR FIRST CLASS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail or as Express Mail with the number of the Express Mail mailing label noted below in an envelope addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231, on APR 3 - 2000 (Date of Deposit)

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The Firm of Karl F. Ross P.C.

21437

IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	Bernd EIKMANNS et al
Patent App.	Not known (US Nat'l phase of PCT/EP98/06210)
Filed	Concurrently herewith
For	METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE SPARTATE AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METH

Hon. Commissioner of Patents
Washington, DC 20231

RECORD OF TRANSMITTAL--PCT APPLICATION

- ☑ PCT Transmittal
- ☑ PCT Application
- ☑ Translation
- ☑ Sheets of Drawing (2)
- ☑ PCT Declaration
- ☑ PCT Documents
- ☑ International Search Report
- ☑ Preliminary Amendment
- ☑ Assignment (with PTO-1595 and sep. check)
- ☑ Reference(s) with PTO-1449
- ☑ Check for Official Fees
- ☑ Basic Fee (Large Entity) $970.00
  - ☐ Ind. claims in excess of 3 $00.00
  - ☑ 17 claims in excess of 20 $306.00

Total **$1276.00**

Please charge any fees not covered by an enclosed check to account 18-2025 of the undersigned.

3 April 2000

Respectfully submitted,
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IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	**Bernd EIKMANNS et al**
Patent App.	**Not known (US Nat'l phase of PCT/EP98/06210)**
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For	**METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE SPARTATE AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METH**
Art Unit	**Not known**

Hon. Commissioner of Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Prior to examination of the above-identified application, please amend as follows:

In the Claims:

Claim 3, line 1, delete "or 2",

Claim 6, line 1, delete "or 5",

Claim 8, line 1, delete "or 7",

Claim 9, line 1, delete "to 8",

Claim 10, line 1, delete "to 9",

Throughout claims 11, 12, 13, 15 and 17, line 1, delete "one of the preceding claims, insert instead -- claim 1 --,

Claim 20, line 2, delete "or 19",

Claim 21, line 2, delete "or 19",

Claim 23, lines 1 and 2, delete "one of claims 18 to 20", insert instead -- claim 18 --,

Claim 24, line 2, delete "one of claims 18 to 23", insert instead -- claim 18 --,

Claim 25, line 2, delete "according to one of claims 18 to 23", line 3, delete "24", insert instead -- 18 --,

Claim 26, line 2, delete "according to one of claims 18 to 23", line 3, delete "24", insert instead -- 18 --,

Claim 27, line 1, delete "according to claim 26",

Claim 28, line 1, delete "or 27",

Claim 29, lines 1 and 2, delete "one of claims 26 to 28", insert instead -- claim 26 --,

Claim 30, lines 1 and 2, delete "one of claims 26 to 29", insert instead -- claim 26 --,

Claim 31, lines and 2, delete "one of claims 26 to 30", insert instead -- claim 30 --,

Claim 34, line 1, delete "or 33",

Claim 36, line 1, delete "one of claims 32 to 35", insert instead -- claim 32 --,

Claim 37, line 1, delete "one of claims 32 to 36", insert instead -- claim 32 --.

This preliminary amendment is submitted just to reduce claim charges.

Respectfully submitted,
The Firm of Karl F. Ross P.C.

By: Herbert Dubno, Reg. No. 19,752
Attorney for Applicant

30 March 2000
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) ss:
County of the Bronx)

TRANSLATOR'S AFFIDAVIT

I, Herbert Dubno, a citizen of the United States of America, residing in Riverdale (Bronx), New York, depose and state that:

I am familiar with the English and German languages;

I have read a copy of the German-language document attached hereto, namely PCT/EP98/06210; and

The hereto-attached English-language text is an accurate translation of the above-identified German-language document.

Herbert Dubno

Sworn to and subscribed before me
23 March 2000

Elise Friedman
Elise Friedman
Notary Public

[T R A N S L A T I O N]

D E S C R I P T I O N

METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE ASPARTATE AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METHOD

The invention relates to a method of microbial production of amino acids of the aspartate family and/or of the glutamate family according to claims 1 to 17, to the pyruvate-carboxylase gene according to claims 18 to 23, gene structures according to claim 24, vectors according to claim 25, transformed cells according to claims 26 to 31 as well as to uses according to claims 32 to 37.

Amino acids are of considerable economic interest since amino acids have many uses: thus, for example, L-lysine and L-threonine, L-methionine and L-tryptophan are necessary as fodder additives, L-glutamate as an additive to suppress L-isoleucine and L-tyrosine in the pharmaceutical industry, L-arginine and L-isoleucine as medicaments or L-glutamate, L-aspartate and L-phenylalanine as starting substances for the synthesis of fine chemicals.

A preferred method of producing these different amino acids is the biotechnical production by means of microorganisms such that in this manner the biologically-effective and optically-active forms of the respective amino acids are obtained and simple

and inexpensive raw materials can be used. As microorganisms, for example, *Corynebacterium glutamicum* and its derivatives *ssp. Flavum* and *ssp. Lactofermentum* (Liebl et al., Int J System Bacteriol 1991, 41: 255 to 260) in addition to *Escherichia coli* and related bacteria are used. These bacteria normally produce the amino acids but only in amounts required for growth so that no surplus amino acids are formed and can be recovered. This is because in the cells the biosynthesis of amino acids is controlled in many ways. As a consequence, there are already known various processes to increase the product formation by cutting out the control mechanisms. In these processes, for example, amino acid analogs are introduced to switch off the effective regulation of the biosynthesis. For example, a process has been used which is resistant to L-tyrosine analogs and L-phenylalanine analogs (JP 19037/1976 and 39517/1978). The processes also have been described in which bacteria resistant to L-lysine analogs or L-phenylalanine analogs have been used to suppress the control mechanisms (EP 0 205 849, GB 2 152 509).

Furthermore, microorganisms which have been constructed also by recombinant DNA-technique which also obviate regulation of biosynthesis in that the gene which is coded in the no-longer feedback-inhibited key enzyme is cloned and expressed. For example, the recombinant L-lysine-producing bacterium with plasmid-coded feedback-resistant aspartate kinase is known (EP 0 381 527). In addition, a recombinant L-phenylalanine-producing bacterium with

feedback-resistant prephenate dehydrogenase is described (JP 123475/1986, EP 0 488 424).

In addition, by overexpression of genes which do not code for feedback-sensitive enzymes as amino acid synthesis, increased amino acid yields are obtainable. thus, for example, a lysine formation can be improved by increased synthesis of the dihydrodipicolinate synthesis (EP 0 197 335). Increasingly, by increased synthesis of the threoninedehydratease, improved isoleucine formation is achieved (EP 0 436 886).

Further investigations in increasing amino acid production have been targeted on the improved availability of the cellular primary metabolites of central metabolism. Thus it is known that, by recombinant techniques, over-expression of the transketolase can bring about an improved product formation of L-tryptophan or L-tyrosine or L-phenylalanine (EP 0 600 463). Furthermore, the reduction of the phosphoenolpyruvate-carboxylase activity in Corynebacterium leads to improved formation of aromatic amino acids (EP 0 3331 145) whereas by contrast the increase in the phosphoenolpyruvate-carboxylase activity in Corynebacterium leads to increased separation out of amino acids of the aspartate family (EP 0 358 940).

During the growth and especially under amino acid production conditions, the tricarboxylic acid cycle must continuously and effectively be supplemented with C4 compounds, for example, oxalic acetate to replace intermediate products withdrawn for the amino acid biosynthesis. Until recently it has been

thought that phosphoenolpyruvate-carboxylase was answerable for these so-called anaplerotic functions in Corynebacterium (Kinoshita, Biology of Industrial Micro-organisms 1985: 115 to 142, Benjamin/Cummings Publishing Company, London; Liebl, The Prokaryotes II, 1991 to 1171, Springer Verlag N.Y.; Vallino and Stephanopoulos, Biotechnol Bioeng 1993, 41: 633 to 646).

It has, however, now been found that phosphoenolpyruvate-carboxylase-negative mutants grow equally by comparison to the respective starting strains on all media (Peters-Wendisch et al., FEMS Microbiology Letters 1993, 112: 269 to 274; Gubler et al., Appl Microbiol Biotechnol 1994, 40: 857 to 863). These results indicate that the phosphoenolpyruvate-carboxylase is not essential for the growth and plays no role or only a small role for the anaplerotic reactions. Furthermore the aforementioned results indicate that in Corynebacterium another enzyme must be provided which is answerable for the synthesis of oxalacetate which is required for the growth. Recently, indeed, a pyruvate-carboxylase activity has been found in permeablized cells of *Corynebacterium glutamicum* (Peters-Wendisch et al., Microbiology 1997, 143: 1095 to 1103). This enzyme is effectively inhibited by AMP, ADP and acetyl coenzyme A and in the presence of lactate as a carbon source is formed in increased quantities. Since one must conclude that this enzyme is answerable primarily for the satisfaction of the tricarboxylic acid cycle of growth, it was to be expected that an increase in the gene expression or the enzymatic activity would either give rise to no increase in the amino acids belonging to the

aspartate or yield only an increase therein. Furthermore, it was to be expected that an increase in the gene expression or the enzymatic activity of the pyruvate-carboxylase would also have no influence on the production of amino acids of other families.

It has surprisingly been found that an increase in the pyruvate-carboxylase activity by genetic modification of the enzyme and/or by increasing the pyruvate-carboxylase gene expression, the microbial production of amino acids of the aspartate and/or the glutamate families can be increased. It has been found that especially strains with increased copy numbers of the pyruvate-carboxylase gene can produce about 50% more lysine, 40% more threonine and 150% more homoserine in the culture medium. It has been found further that, surprisingly, the glutamate production is also significantly increased (compare especially the example under 6. Table 4).

The genetic alteration of the pyruvate-carboxylase to increase the enzyme activity is effected preferably by mutation of the endogenous gene. Such mutation can either be achieved by classical methods like, for example, by UV irradiation or by mutation triggering the chemicals or targeted by means of gene technological methods like deletion, insertion and/or nucleotide exchange.

The pyruvate-carboxylase gene expression is increased by increasing the gene copy number and/or by reinforcing regulatory factors which positively influence the expression of the gene. Thus a reinforcement of regulatory elements, preferably on the

transcription plane can be effected in that especially the transcription signals are increased. This can be effected, for example, by varying the promoter sequence of the promoter preceding the structure gene to enhance its effectiveness or by replacing the promoter completely by more effective promoters. A reinforcement of the transcription can also be effected by a corresponding influence on a regulator gene associated with the pyruvate-carboxylase gene. This can be achieved, for example, by mutation of a regulatory gene sequence to influence the effectivity of the binding of a regulator protein to the DNA of the pyruvate-carboxylase gene which is regulated so that the transcription is thereby enhanced and thus the gene expression is increased. Furthermore the pyruvate-carboxylase gene can also be associated with a so-called "enhancer" as a regulatory sequence and which by means of an improved interchange between RNA polymerase and DNA also effects an increased pyruvate-carboxylase gene expression. However, a reinforcement of translations is also possible in that, for example, the stability of the m-RNA is improved.

For increasing the gene copy number the pyruvate-carboxylase gene is built into a gene construct or vector. The gene construct contains especially the regulatory sequences associated with the pyruvate-carboxylase gene, preferably those which reinforce the gene expression. For the incorporation of the pyruvate-carboxylase gene in a gene construct, the gene is progressively isolated from a microorganism strain of the Corynebacterium variety and is transformed in an amino-acid

producing microorganism strain, especially Corynebacterium or in *Escherichia coli* or serratia marcenscens. For the process of the invention, especially genes from *C. glutamicum* or *C. glutamicum* ssp. flavum or *C. glutamicum* ssp. lactofermentum are suitable. After isolation of the gene and in the in vitro recombination with known vectors (see for example Simon et al., Bio/Technology 1983, 1: 784 to 791; Eikmanns et al., Gene 1991, 102: 93 to 98), the transformation is effected in the amino-acid producing strain by electroporation (Liebl et al., FEMS Microbiology Letters 1991, 65: 299 to 304) or conjugation (Schäfer et al., J. Baceriol 1990, 172: 1663 to 1666).

As the host strain preferably such amino-acid producers are used which have been deregulated in the synthesis of the corresponding amino acid and/or show an increased export carrier activity for the corresponding amino acid. Furthermore, such strains are preferred which contain an increased number of such central metabolism metabolites as anticipated in the synthesis of the corresponding amino acid and/or strains which contain a reduced proportion of the central metabolism metabolites which do not participate in the synthesis of the corresponding amino acid, especially metabolites which tolerate competitive reactions; i.e. such strains are preferred with which by synthesis paths competitive with the corresponding amino acid biosynthesis path runs with reduced activity. Thus, especially a *Coryne*-former microorganism strain with reduced citrate synthase activity is

suitable as a strain resistant to L-asparaginic-acid-β-methylester (AME) is suitable (EP 0 551 614).

After isolation, the pyruvate-carboxylase gene is obtained with nucleotide sequences which code for the amino acid sequence given under SEQ ID No. 2 or their allele variations or the nucleotide sequence of nucleotides 165 to 3587 according to SEQ ID No. 1 or a substantially identically-effective DNA sequence. The gene further contains a protein promoter of the nucleotide sequence of nucleotides 20 to 109 according to SEQ ID No. 1, a substantially identically effective DNA sequence. Allele variations or identically effective DNA sequences encompass especially functional derivations which are corresponding nucleotide sequences formed by deletions, insertions and/or substitutions of nucleotides whereby the enzyme activity or function remains or can even be increased. This pyruvate-carboxylase gene is preferably used in the process of the invention.

The pyruvate-carboxylase gene with or without the preceding promoter or with or without the associated regulator gene can be preceded by and/or followed by one or more DNA sequences so that the gene is contained in a gene structure.

The pyruvate-carboxylase gene is preferably preceded by the tac-promoter ($lacI^q$-Gen) with which is associated especially regulatory sequences.

By cloning the pyruvate-carboxylase gene, plasmids are obtained which contain the gene and are suitable for transformation to an amino acid producer. The cells obtained by transformation

which preferably correspond to transformed cells of Corynebacterium, contain the gene in replicatable form, i.e. in additional copies on the chromosome, whereby the gene copies are integrated by recombination at optional sites in the genome and/or on a plasmid or vector.

Example

1. Cloning the Pyruvate-Carboxylase Gene of *Corynebacterium Glutamicum*

Starting from conserved regions of all prior known pyruvate-carboxylase-(pyc-) genes of *Saccharomyces cerevisiae* (J Biol Chem 1988, 263: 11493-11497; Mol Gen Genet 1991, 229: 307-315), Mensch (Biochem Biophys Acta 1994, 1227: 46-52), Maus (Proc Natl Acad Sci, USA 1993, 90: 1766-1770), Aedes aegypti (EMBL-GeneBank: Accession Nr. L36530) and from Mycobacterium tuberculosis (EMBL-GeneBank: Accession Nr. U00024), PCR primer is synthesized (MWG Biotech). The primer corresponds to the bases 810 to 831 and 1015 to 1037 of the pyc gene from M. tuberculosis. With this primer, by means of PCR according to the standard method of Innis et al (PCR protocols. A Guide to Methods and Applications, 1990, Academic Press) for nongenerated homologous primer, in a fragment of about 200 bp of chromosomal DNA of *C. glutamicum* ATCC 13032 as has been described by Eikmanns et al. (Microbiology 1994, 140: 1817-1828) is isolated following amplification. The size of 200 bp corresponds to the expectation for the pyc gene. The PCR product as described by Sanger et al (Proc Natl Acad Sci USA 1977, 74:

5463-5467) was sequenced. The sequencing was carried out with fluorescence-marked ddNTPs with an automatic DNA sequencing apparatus (Applied Biosystems).

Starting from this DNA fragment of *C. glutamicum*, the following homologous oligonucleotides are produced:

pyc 1 5'- CGTCTTCATCGAAATGAAC-3'

pyc 2 5'- ACGGTGGTGATCCGGCACT-3'

The oligonucleotide is used as a PCR primer for isolating the probe for the gene of pyruvate-carboxylase (pyc) from *C. glutamicum*. The primer is introduced into a PCR reaction with chromosomal DNA from *C. glutamicum* and digoxygenine-marked nucleotides. The reaction is carried out in accordance with the instructions of the "PCR DIG Labeling Kits" of the firm Boehringer Mannheim. With this approach, a digoxygenine-marked DNA fragment is amplified which corresponds to the expected size of about 200 bp. The thus produced pyc probe is then used to identify, utilizing Southern-blot-hybridization, A DNA fragment in the chromosomal DNA of *C. glutamicum* on which the pyc gene is localized. For this purpose each 2 to 5 µg of chromosomal DNA from *C. glutamicum* WT is cleaved with the restriction enzyme HindIII, SphI, SalI, DdraI, EcoRI and BamHI and the obtained DNA fragments are correspondingly separated by size over 16 hours at 20 volts gel-electrophoretically in an 0.8% agarose gel. The DNA fragments found in the agarose gel are denatured by the Southern blot (J Mol Biol 1975, 98: 503-517) and subjected to the vacuum-supported separation with the VacuGene Blot Apparatus of Pharmacia LKB

(Uppsala, Sweden) from the gene matrix transferred onto a nylon membrane (Nytran N13 of Schleicher and Schüll, Dassel, Switzerland), immobilized and the digoxygenine marker detected by means of NBT/X phosphate conversion with alkali phosphatizes in this manner. Following chromosomal fragments hybridized with the pyc-DNA-probe can be detected: a 17 kb HindIII-fragment, a 6.5 kb SalI fragment and a 1.35 kb EcoRI fragment.

The 17 kb HindIII fragment was isolated and subcloned. For this purpose a cosmid gene bank of chromosomal DNA from *C. glutamicum* in cosmid pH C79 was used which represented the genome of *C. glutamicum* to 99% (Mol Microbiol 1992, 6: 317-326). The *E. coli* strain DH5α was transformed with this gene bank by means of the $CaCl_2$ method of Sambrook et al (Molecular Cloning, A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press) and plated out to about 300 colonies per LB-agar plate with 50 µg/l kanamycin (a total of 5000 colonies). Then the obtained transformed product was transferred on a nytran N13 filter and incubated for 5 minutes for alkali lysis of the cells and denaturing of the DNA on Whatmann paper soaked with 0.5 M NaOH and 1.5 M NaCl. The subsequent neutralization is effected with 1 M Tris/HCl pH 7.5 and 1.5 M NaCl. The subsequent neutralization is effected with 1 M Tris/HCl pH 7.5 and 1.5 M NaCl.

After incubation of the filter in 2 x SSC, the liberated DNA is fixed by UV radiation at 366 nm on the filter. Then the remaining cell fragments are removed by shaking in 3 x SSC, 0.1% SDS at 50°C. The filter in this form is used for the hybdridiza-

tion with a specific pyc probe as described by Southern (J Mol Biol 1975, 98: 503-517).

The 3 transformands were identified from the pyc probe hybridization. From these transformands the cosmid DNA was isolated by means of plasmid proportion in accordance with the alkali lysis method of Birnboim (Meth Enzymol 1983, 100: 243-255) and then tested by restriction and Southern blot analysis for the presence of the HINDIII fragments. The cosmid pH C79-10 which contains a 40 kb HINDIII transmission completely and was further analyzed. It showed that also after the restriction with the endonucleosis SaII and EcoRI the same hybridized fragments as in the chromosomal DNA, i.e. a 6.5 kb SalI- fragment and a 1.35 kb EcRI-fragment. The 17 kb HindIII-fragment was isolated by restriction from the cosmid and is ligated in the *E. coli* vector pUC 18, which is also cleaved with HindIII. A restriction analysis of the fragments in the resulting vector pUC pyc was carried out. The physical mapping of the fragments is shown in FIG. 1.

2. Sequencing of the Pyruvate-Carboxylase Gene

In further subcloning steps a 0.85 kb SalI-EcoRI-fragment was isolated from the plasmid pUC pyc by restriction with corresponding restriction enzymes as a 1.35 kb EcoRI-fragment, a 1.6 kb EcoRI-EcoRI-StuI-fragment as well as a 1.6 kb ClaI-fragment, that overlapped with 0.85 kb SalI-EcoRI-fragment. By ligation the fragments were cloned correspondingly in the restricting vector pUC 18 and then sequenced as described above according to Sanger et al.

In (Proc Natl Acad Sci USA 1977, 74: 5463-5467) the nucleotide sequences obtained were analyzed. The program package HUSAR (Release 3.0) of the German zone for cancer research (Heidelberg). The sequence analysis of the fragments gave a continuously open reading raster of 3576 bp which coded for a protein sequence of 1140 amino acids. Comparison of the protein sequence with the EMBL gene data bank (Heidelberg) gave similarities to all known pyruvate carboxylases. The highest identity (62%) was to the putative pyruvate-carboxylase from *Mycobacterium tuberculosis* (EMBL-GeneBank: Accession No. U00024). The similarity amounted to 76% when conserved amino acid exchange was followed. A comparison with the pyruvate-carboxylase of other organisms yielded an identity of 46 to 47% identical and 64 to 65% similar amino acids (Gene 1997, 191: 47-50; J Bacteriol 1996, 178: 5960-5970; Proc Natl Acad Sci USA 1993, 990: 1766-1770; Biochem J 1996, 316: 631-637; EMBL-GenBank: Accession No. L36530; J Biol Chem 1988, 263: 11493-11497; Mol Gen Genet 1991, 229: 307-315). From these results it could be concluded that the cloned fraction base was the gene for the pyruvate-carboxylase from *C. glutamicum*. The nucleotide sequence of the gene is given under SEQ ID No. 1 and the corresponding amino acid sequence under SEQ ID No. 2.

3. Overexpression of the Pyruvate-Carboxylase

For the overexpression of the gene for pyruvate-carboxylase from *C. glutamicum*, the gene was cloned from the plasmid pUCpyc as the 6.2 kb Sspl-Scal-fragment in the *E. coli*

glutamicum swing vector pEK0 (Gene 1991, 102: 93-98) which was cleaved with the restriction endonucleosis EcoRI and PstI. By means of Klenow-polymerase treatment the overhanging ends were ligated to smooth ends by filling the EcoRI or linking PstI and the linearized vector was ligated with the 6.2 kb Sspl-Scal-fragment. The resulting construct pEK0pyc was additionally transformed in the *E. coli* strain DH5α, the plasmid DNA was isolated on the resulting transformand and the correctness of the inserts controlled by restriction. The DNA was then introduced in the strain SP 733 by electroporation (FEMS Microbiol Lett 1989, 65: 299-304).

This strain is a mutant of the restriction negative *C. glutamicum* strain R 127 (Dechema Biotechnology Conference 1990, 4: 323-327, Verlag Chemie) which was obtained by chemical mutagenesis and was characterized in that it cannot be grown on a minimal medium with pyruvate and lactate as single carbon sources (Microbiology 1997, 143: 1095-1103). This phenotype is recognized as a defect in the pyruvate-carboxylase and can be complemented by introducing the pyruvate-carboxylase gene from *C. glutamicum*, i.e. the strain which is carried by the plasmid pEK0pyc and was by contrast to the starting strain able to grow again in the presence of minimal medium with lactate as a single carbon source. This was a verification that the gene was coded for a functional pyruvate-carboxylase

Furthermore, the plasmid pEK0pyc was transformed in the *C. glutamicum* wild type ATCC 13032 by electroporation. The resulting strain WT (pEK0pyc) was investigated by comparison to the

wild type ATCC 13032 with respect to its pyruvate-carboxylase activity. The strain was cultured in a complex medium (Luria-Bertani, Molecular Cloning, A laboratory manual, 1989, Cold Spring Harbour Laboratory Press) with 0.5% lactate and on minimal medium with 2% lactate or 4% glucose and the pyruvate-carboxylase test was carried out corresponding to the method as described by Peters-Wendisch et al (Microbiology 1997, 143: 1095-1103). The results of the analysis (Table 1) showed that the pyruvate-carboxylase activity in the pEK0-pyc-carrying strain was about 4 times higher than in the starting strain.

4. Increased Accumulation of Lysine by Overexpression of the Pyruvate-Carboxylase Gene in the Strain *C-glutamicum* DG 52-5.

To investigate the effect of the overexpression of the gene for the pyruvate-carboxylase in the lysine-producing strain DG 52-5 (J Gen Microbiol 1988,134: 3221-3229), the expression vector pVWEX1 is used to promote an IPTG-inducible expression. IN this vector, the pyc gene was promotorlessly cloned. For that purpose, initially PCT-Primer (Primer 1 = Postion 112 - 133; Primer 2 = Position 373 to 355 in the nucleotide sequence according to SEQ ID No. 1), is synthesized and 261 bp of the promotorless starting region of the pyruvate-carboxylase gene was amplified by means of PCR. The primer was so selected that Primer I enabled a PstI cleavage site and Primer 2 a BamHI cleavage site. After the PCR, the 274 bp PCR product was isolated, ligated to concatemers and then cleaved with the restriction enzymes PstI and BamHI. The

restriction product was concentrated by ethanol precipitation and then ligated with the PstI-BamHI cleaved vector pVWEX1. The resulting construct pVWEXi-PCR was tested by restriction. The end region of the pyc gene was isolated by RcaI-Klenow-SalI treatment from the vector pEK0pyc and ligated in the BamHI-Klenow-SalI during vector PVWEX1-PCR. The resulting construct pVWEX1pyc was analyzed by restriction mapping. Physical mapping of the plasmid is shown in FIG. 2.

The plasmid was introduced by electroporation in the *C. glutamicum* strain DG 52-5. As a control, the strain DG 52-5 was transformed with the vector pVWEX1 without insert and the L-lysine precipitation of three different transformands were compared. For this purpose (DG 52-5 (pVWEX1pyc) 3,4 and (2xTY; Molecular Cloning, A laboratory manual, 1989, Cold Spring Harbour Laboratory Press with 50 µg/I kanamycin) and the respective fermentation medium in each case from the preculture was separately inoculated. The medium contained additional kanamycin to maintain the plasmid stable. In each case two parallel tests were run whereby one flask of 200 µg IPTG/ml was added while the second flask contained no IPTG. After cultivation for 48 hours at 30°C on a rotation shaker at 120 RPM, the accumulated lysine quantity in the medium was determined. The determination of the amino acid concentration was effected by means of high-pressure liquid chromatography (J Chromat 1983, 266; 471-482).

The results of the fermentation are shown in Table 2 whereby the values given are mean values each form three

experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene results in a 50% increased accumulation of lysine in the medium. Thus the use of the covered and described gene for the anapleurotic enzyme pyruvate-carboxylase enables a process of lysine formation to be significantly improved.

5. Increased Accumulation of Threonine and Homoserine by Overexpression of the Pyruvate-Carboxylase Gene in the Strain *C. glutamicum* DM 368-3

Analogously to the experiment in L-lysine formation, the accumulation of threonine in the culture supernatant by overexpression of the gene for pyruvate-carboxylase was also investigated for this purpose, as has been described under point 4, the threonine production strain *C. glutamicum* DM 368-3 (Degussa AG) was transformed with the plasmid pVWEX1pyc with control by the plasmid pVWEX1 and the threonine separation was investigated with each of three different transformands. For this purpose DM 368-3 (pVWEX1) 2 and 3 and DM 368-3 (pVWEX1pyc) 1, 2 and 3 in complex medium (2xTY with 50 µg/l kanamycin) were cultured and the fermentation medium CGXII (J Bacteriol 1993, 175: 5595-5603) in each case was separately inoculated from the preculture. The medium contained additional kanamycin to hold the plasmid stable. Two parallel sets of tests were carried out whereby 200 µg IPTG/ml was added to one flask while the second flask contained no IPTG. After culturing for 48 hours at 30°C on a rotation shaker at 120

RPM, the threonine quantities accumulated in the medium were determined. The determination of the amino acid concentration was effected also by means of high-pressure liquid chromatography (J Chromat 1983, 266: 471-482). The results of the fermentation are shown in Table 3 whereby the values given are mean values from each of three experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene gave about a 40% increase in the threonine concentration in the medium. The use of the covered and described gene for anapleurotic enzyme pyruvate-carboxylase in a process for L-threonine formation significantly improves the latter.

Furthermore, the amino acid concentration determination shows surprisingly that the strain with the overexpressed pyruvate-carboxylase gene also yields 150% more homoserine in the medium than the strain with the nonoverexpressed gene. Corresponding results are shown in Table 3. They make clear that in the process according to the invention the threonine like the homoserine can be significantly improved.

6. Increased Accumulation of Glutamate by Overexpression of the Pyruvate-Carboxylase Gene in *C. glutamicum* Wild Type

Analogous to the experiments for L-lysine, L-threonine and L-homoserine formation (see above, the 4. and 5.), accumulation of glutamate in the culture supernatant, overexpression of the gene for pyruvate-carboxylase was also investigated. For this purpose, as described, the point 4 wild type *C-glutamicum* ATCC 13032 with

the plasmid pVWEX1 pyc was transformed in addition to the control with the plasmid pVWEX1 and the glutamate separation determined from each of two different transformands. Thus *C. glutamicum* ATCC 13032 pVWEX1pyc) D1 and D2 as well as *C. glutamicum* ATCC 13032 (pVWEX1 pyc) 1 and 2 were cultured in the complex medium (2xTY with 50 µg/l kanamycin) and the fermentation medium CGXII (J Bacteriol 1993, 175: 5595-5603) in each case was separately inoculated from the preculture period. The medium contained additional kanamycin to stabilize the plasmid. To induce glutamate separation, 25 mg Tween 60 was added per ml to the medium about 6 hours after the inoculation. Two parallel sets of tests were carried out whereby in one, 200 µg IPTG/ml is added to the flask while the second flask contained no IPTG. After culturing for 48 hours at 30°C on a rotation shaker at 120 RPM, the glutamate quantity accumulated in the medium was determined. The determination of the amino acid concentration was effected also by means of high-pressure liquid chromatography (J Chromat 1983, 266; 471-482). The results of the fermentation are shown in Table 4 whereby values given are averages with each two experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene gave rise to up to 500% increase of the glutamate concentration in the medium. The use of the covered and described gene for the anapleurotic enzyme pyruvate-carboxylase improved the glutamate formation significantly.

Strain	IPTG [µg/ml]	Pyruvat-Carboxylase [nmol min^{-1} mg Dry Weight $^{-1}$]
13032(pEK0pyc)	0	75 ± 13
ATCC 13032	0	19 ± 4
DG52-5(pVWEX1pyc)	200	88 ± 13
	0	11 ± 2
DG52-5(pVWEX1)	200	5 ± 2
	0	6 ± 1
DM368-3(pVWEX1pyc)	200	76 ± 10
	0	12 ± 3
DM368-3(pVWEX1)	200	10 ± 1
	0	11 ± 2

Table 1

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Strain	IPTG [µg/ml]	Lysine [mM]
DG52-5(pVWEX1pyc)	200	35.4 ± 2.6
	0	23.6 ± 2.9
DG52-5(pVWEX1)	200	23.3 ± 2.9
	0	22.1 ± 4.0

Table 2

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Strain	IPTG [µg/ml]	Threonine [mM]	Homoserine [mM]
DM368-3(pVWEX1pyc)	200	10,2 ± 0.5	14,4 ± 1,2
	0	7,9 ± 1.0	5,6 ± 0,2
DM368-3(pVWEX1)	200	8,0 ± 0,5	5.8 ± 0,7
	0	7.5 ± 0,8	6,1 ± 1,0

Table 3

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Strain	IPTG [µg/ml]	Glutamate [mM]
ATCC 13032	200	11 ± 2
ATCC 13032	0	13 ± 2
ATCC 13032(pVWEX1-pyc)	200	67 ± 4
ATCC 13032(pVWEX1-pyc)	0	32 ± 4

Table 4

Patent Claims

1. A method of microbial production of amino acids of aspartate and/or glutamate families in which the pyruvate-carboxylase activity is increased by genetic modification of the enzyme and/or the pyruvate-carboxylase gene expression of the corresponding amino-acid-producing micro organism.

2. The method of claim 1, characterized in that, by mutation of the endogenous pyruvate-carboxylase gene an enzyme with higher pyruvate-carboxylase activity is produced.

3. The method of claim 1 or 2, characterized in that, the gene expression of the pyruvate-carboxylase is increased by increasing the gene copy number.

4. The method according to claim 3, characterized in that, to increase the gene copy number the pyruvate-carboxylase gene is incorporated in a gene construct.

5. The method according to claim 3, characterized in that, the gene is incorporated in a gene construct which contains regulatory gene sequences associated with the pyruvate-carboxylase gene.

6. The method according to claim 4 or 5, characterized in that, the corresponding amino-acid-producing microorganism is transformed with the gene-containing gene construct.

7. The method according to claim 6, characterized in that, a microorganism of the species *Corynebacterium* is transformed with the gene containing the gene construct.

8. The method according to claim 6 or 7, characterized in that, for the transformation a microorganism is used in which the enzyme participating in the synthesis of the corresponding amino acid is deregulated and/or wherein an enhanced export carrier activity is shown for the corresponding amino acid.

9. The method according to claim 6 to 8, characterized in that, for the transformation a microorganism is used which has a higher proportion of the central metabolism metabolites of the corresponding amino acid participating in the synthesis.

10. The method according to claim 6 to 9, characterized in that, for the transformation a microorganism is used in which biosynthesis paths competing with the corresponding amino acid biosynthesis paths runs with reduced activity.

11. The method according to one of the preceding claims, characterized in that, the pyruvate-carboxylase gene is isolated from a microorganism strain of the variety *Corynebacterium*.

12. The method according to one of the preceding claims, characterized in that, the gene expression is increased by reinforcement of the transcription signal.

13. The method according to one of the preceding claims, characterized in that, the pyruvate-carboxylase gene has the tac-promoter ahead of the pyruvate-carboxylase gene.

14. The method according to claim 13, characterized in that, the tac-promoter is associated with regulatory sequences.

15. The method according to one of the preceding claims, characterized in that, the pyruvate-carboxylase gene is a gene with the amino acid sequence given under SEQ ID No. 2 and its allele variation coding nucleotide sequences.

16. The method according to claim 15, characterized in that, with the pyruvate-carboxylase gene a gene with the nucleotide sequence of nucleotide 165 to 3587 according to SEQ ID No. 1 or a substantially identically-effective DNA sequence is used.

17. The method according to one of the preceding claims for the production of lysine, threonine, homoserine, glutamate and/or arginine.

18. A pyruvate-carboxylase gene coding for the amino acid sequence given under SEQ ID No. 2 and /or a nucleotide sequence coding for its allele variations.

19. The pyruvate-carboxylase gene according to claim 18 with the nucleotide sequence of nucleotides 165 to 3587 according to SEQ ID No. 1 or a substantially identically-effective DNA sequence.

20. The pyruvate-carboxylase gene according to claim 18 or 19 with a preceding promoter of the nucleotide sequence from nucleotide 20 to 109 according to SEQ ID No. 1 or a substantially-identically-effective D NA sequence.

21. The pyruvate-carboxylate gene according to claim 18 or 19, with preceding tac-promoter.

22. The pyruvate-carboxylase gene according to claim 21 with the regulatory sequence associated with the promoter.

23. The pyruvate-carboxylase gene according to one of claims 18 to 20 with these regulatory gene sequences associated therewith.

24. A gene structure containing a pyruvate-carboxylase gene according to one of claims 18 to 23.

25. A vector containing a pyruvate-carboxylase gene according to one of claims 18 to 23 or a gene structure according to claim 24.

26. Transformed cells containing in replicatable form a pyruvate-carboxylase gene according to one of claims 18 to 23 or a gene structure according to claim 24.

27. Transformed cells according to claim 26 containing a vector according to claim 25.

28. Transformed cells according to claim 26 or 27, characterized in that, they belong to the variety *Corynebacterium*.

29. Transformed cells according to one of claims 26 to 28, characterized in that, enzymes which participate in the synthesis of the corresponding amino acid and/or enzyme which participate in the export of the corresponding amino acid are deregulated.

30. Transformed cells according to one of claims 26 to 29, characterized in that, they contain an increased proportion of the central metabolism metabolites participating in the synthesis of the corresponding amino acid.

31. Transformed cells according to one of claims 26 to 30, characterized in that, they contain a reduced proportion of the central metabolism metabolites which do not participate in the synthesis of the corresponding amino acid.

32. The use of a pyruvate-carboxylase gene for increasing the production of amino acids of the aspartate and/or glutamate families by microorganisms.

33. The use according to claim 32, characterized in that, a mutated pyruvate-carboxylase gene which codes for an enzyme with increase pyruvate-carboxylase activity is used.

34. The use according to claim 32 or 33, characterized in that, the microorganism producing the corresponding amino acid

is transformed with a gene construct that contains a pyruvate-carboxylase gene.

35. The use according to claim 34, characterized in that, the gene construct additionally contains regulatory gene sequences.

36. The use according to one of claims 32 or 35, characterized in that, a pyruvate-carboxylase gene from *Corynebacterium* is used.

37. The use according to one of claims 32 or 36, characterized in that, *Corynebacterium* is used as the amino acid-producing microorganism.

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SEQUENCE PROTOCOL

(1) GENERAL DETAILS :

(i)APPLICANTS
(A) NAME: Forschungszentrum Juelich GmbH
(B) STREET: Postfach 1913
(C) LOCALE: Juelich
(E) COUNTRY: GERMANY
(F) ZIP CODE : 52425

(ii)DESIGNATION OF THE INVENTION: Pyruvate Carboxylase

(iii)NUMBER OF SEQUENCES: 2

(iv) COMPUTER-READABLE FORM :
(A) DATA CATEGORY: floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPA)

(2) DETAILS TO SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3728 Base PAIRS
(B) TYPE: Nucleotide
(C) STRAND SHAPE: Single strand
(D) TOPOLOGY : linear

(ii)TYPE OF MOLECULES : Genom DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGCAACCGTG CTTGAAGTCG TGCAGGTCAG GGGAGTGTTG CCCGAAAACA TTGAGAGGAA 60

AACAAAAACC GATGTTTGAT TGGGGGAATC GGGGGTTACG ATACTAGGAC GCAGTGACTG 120

CTATCACCCT TGGCGGTCTC TTGTTGAAAG GAATAATTAC TCTAGTGTCG ACTCACACAT 180

CTTCAACGCT TCCAGCATTC AAAAAGATCT TGGTAGCAAA CCGCGGCGAA ATCGCGGTCC 240

GTGCTTTCCG TGCAGCACTC GAAACCGGTG CAGCCACGGT AGCTATTTAC CCCCGTGAAG 300

ATCGGGGATC ATTCCACCGC TCTTTTGCTT CTGAAGCTGT CCGCATTGGT ACCGAAGGCT 360

CACCAGTCAA GGCGTACCTG GACATCGATG AAATTATCGG TGCAGCTAAA AAAGTTAAAG 420

CAGATGCCAT TTACCCGGGA TACGGCTTCC TGTCTGAAAA TGCCCAGCTT GCCCGCGAGT 480

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ATAAGTCTCG CGCGGTAACC GCCGCGAAGA AGGCTGGTCT GCCAGTTTTG GCGGAATCCA 600

CCCCGAGCAA AAACATCGAT GAGATCGTTA AAAGCGCTGA AGGCCAGACT TACCCCATCT 660

TTGTGAAGGC AGTTGCCGGT GGTGGCGGAC GCGGTATGCG TTTTGTTGCT TCACCTGATG 720

AGCTTCGCAA ATTAGCAACA GAAGCATCTC GTGAAGCTGA AGCGGCTTTC GGCGATGGCG 780

CGGTATATGT CGAACGTGCT GTGATTAACC CTCAGCATAT TGAAGTGCAG ATCCTTGGCG 840

ATCACACTGG AGAAGTTGTA CACCTTTATG AACGTGACTG CTCACTGCAG CGTCGTCACC 900

AAAAAGTTGT CGAAATTGCG CCAGCACAGC ATTTGGATCC AGAACTGCGT GATCGCATTT 960

GTGCGGATGC AGTAAAGTTC TGCCGCTCCA TTGGTTACCA GGGCGCGGGA ACCGTGGAAT 1020

TCTTGGTCGA TGAAAAGGGC AACCACGTCT TCATCGAAAT GAACCCACGT ATCCAGGTTG 1080

AGCACACCGT GACTGAAGAA GTCACCGAGG TGGACCTGGT GAAGGCGCAG ATGCGCTTGG 1140

CTGCTGGTGC AACCTTGAAG GAATTGGGTC TGACCCAAGA TAAGATCAAG ACCCACGGTG 1200

CAGCACTGCA GTGCCGCATC ACCACGGAAG ATCCAAACAA CGGCTTCCGC CCAGATACCG 1260

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CTGGTGTTGC AACCAACATT GGTTTCTTGC GTGCGTTGCT GCGGGAAGAG GACTTCACTT 1500

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ATGGTGTGCG TCCAAAGGAT GTTGCAGCTC CTATCGATAA GCTGCCTAAC ATCAAGGATC 1680

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CGATGCGTTT CCTCTTTGAG GATCCGTGGG ACAGGCTCGA CGAGCTGCGC GAGGCGATGC 1980

CGAATGTAAA CATTCAGATG CTGCTTCGCG GCCGCAACAC CGTGGGATAC ACCCCGTACC 2040

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ACGGTGCTTC CGCACCACTG TCTGGCACCA CCTCCCAGCC ATCCCTGTCT GCCATTGTTG 2520

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AGCCGTACTG GGAAGCAGTG CGCGGACTGT ACCTGCCATT TGAGTCTGGA ACCCCAGGCC 2640

CAACCGGTCG CGTCTACCGC CACGAAATCC CAGGCGGACA GTTGTCCAAC CTGCGTGCAC 2700

AGGCCACCGC ACTGGGCCTT GCGGATCGTT TCGAACTCAT CGAAGACAAC TACGCAGCCG 2760

TTAATGAGAT GCTGGGACGC CCAACCAAGG TCACCCCATC CTCCAAGGTT GTTGGCGACC 2820

TCGCACTCCA CCTCGTTGGT GCGGGTGTGG ATCCAGCAGA CTTTGCTGCC GATCCACAAA 2880

AGTACGACAT CCCAGACTCT GTCATCGCGT TCCTGCGCGG CGAGCTTGGT AACCCTCCAG 2940

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CTCTGACGGA AGTTCCTGAG GAAGAGCAGG CGCACCTCGA CGCTGATGAT TCCAAGGAAC 3060

GTCGCAATAG CCTCAACCGC CTGCTGTTCC CGAAGCCAAC CGAAGAGTTC CTCGAGCACC 3120

GTCGCCGCTT CGGCAACACC TCTGCGCTGG ATGATCGTGA ATTCTTCTAC GGCCTGGTCG 3180

AAGGCCGCGA GACTTTGATC CGCCTGCCAG ATGTGCGCAC CCCACTGCTT GTTCGCCTGG 3240

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AGGCAGATTC CTCCAACAAG GGCCATGTTG CTGCACCATT CGCTGGTGTT GTCACCGTGA 3420

CTGTTGCTGA AGGTGATGAG GTCAAGGCTG GAGATGCAGT CGCAATCATC GAGGCTATGA 3480

AGATGGAAGC AACAATCACT GCTTCTGTTG ACGGCAAAAT CGATCGCGTT GTGGTTCCTG 3540

CTGCAACGAA GGTGGAAGGT GGCGACTTGA TCGTCGTCGT TTCCTAAACC TTTCTGTAAA 3600

AAGCCCCGCG TCTTCCTCAT GGAGGAGGCG GGGCTTTTTG GGCCAAGATG GGAGATGGGT 3660

GAGTTGGATT TGGTCTGATT CGACACTTTT AAGGGCAGAG ATTTGAAGAT GGAGACCAAG 3720

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GCTCAAAG 3728

(2)DETAILS TOSEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A)LENGTH: 1140 Aminosäuren
(B)TYPE: Aminosäure
(C) STRAND SHAPE:single strand
(D) TOPOLOGY: linear

(ii)TYPE OF MOLECULE: Protein

(xi) SEQUENCE DESCRIPTION:SEQ ID NO: 2:

Met Ser Thr His Thr Ser Ser Thr Leu Pro Ala Phe Lys Lys Ile Leu
1 5 10 15

Val Ala Asn Arg Gly Glu Ile Ala Val Arg Ala Phe Arg Ala Ala Leu
20 25 30

Glu Thr Gly Ala Ala Thr Val Ala Ile Tyr Pro Arg Glu Asp Arg Gly
35 40 45

Ser Phe His Arg Ser Phe Ala Ser Glu Ala Val Arg Ile Gly Thr Glu
50 55 60

Gly Ser Pro Val Lys Ala Tyr Leu Asp Ile Asp Glu Ile Ile Gly Ala
65 70 75 80

Ala Lys Lys Val Lys Ala Asp Ala Ile Tyr Pro Gly Tyr Gly Phe Leu
85 90 95

Ser Glu Asn Ala Gln Leu Ala Arg Glu Cys Ala Glu Asn Gly Ile Thr
100 105 110

Phe Ile Gly Pro Thr Pro Glu Val Leu Asp Leu Thr Gly Asp Lys Ser
115 120 125

Arg Ala Val Thr Ala Ala Lys Lys Ala Gly Leu Pro Val Leu Ala Glu
130 135 140

Ser Thr Pro Ser Lys Asn Ile Asp Glu Ile Val Lys Ser Ala Glu Gly
145 150 155 160

Gln Thr Tyr Pro Ile Phe Val Lys Ala Val Ala Gly Gly Gly Gly Arg
165 170 175

Gly Met Arg Phe Val Ala Ser Pro Asp Glu Leu Arg Lys Leu Ala Thr
180 185 190

Glu Ala Ser Arg Glu Ala Glu Ala Ala Phe Gly Asp Gly Ala Val Tyr

195 200 205

Val Glu Arg Ala Val Ile Asn Pro Gln His Ile Glu Val Gln Ile Leu
210 215 220

Gly Asp His Thr Gly Glu Val Val His Leu Tyr Glu Arg Asp Cys Ser
225 230 235 240

Leu Gln Arg Arg His Gln Lys Val Val Glu Ile Ala Pro Ala Gln His
245 250 255

Leu Asp Pro Glu Leu Arg Asp Arg Ile Cys Ala Asp Ala Val Lys Phe
260 265 270

Cys Arg Ser Ile Gly Tyr Gln Gly Ala Gly Thr Val Glu Phe Leu Val
275 280 285

Asp Glu Lys Gly Asn His Val Phe Ile Glu Met Asn Pro Arg Ile Gln
290 295 300

Val Glu His Thr Val Thr Glu Glu Val Thr Glu Val Asp Leu Val Lys
305 310 315 320

Ala Gln Met Arg Leu Ala Ala Gly Ala Thr Leu Lys Glu Leu Gly Leu
325 330 335

Thr Gln Asp Lys Ile Lys Thr His Gly Ala Ala Leu Gln Cys Arg Ile
340 345 350

Thr Thr Glu Asp Pro Asn Asn Gly Phe Arg Pro Asp Thr Gly Thr Ile
355 360 365

Thr Ala Tyr Arg Ser Pro Gly Gly Ala Gly Val Arg Leu Asp Gly Ala
370 375 380

Ala Gln Leu Gly Gly Glu Ile Thr Ala His Phe Asp Ser Met Leu Val
385 390 395 400

Lys Met Thr Cys Arg Gly Ser Asp Phe Glu Thr Ala Val Ala Arg Ala
405 410 415

Gln Arg Ala Leu Ala Glu Phe Thr Val Ser Gly Val Ala Thr Asn Ile
420 425 430

Gly Phe Leu Arg Ala Leu Leu Arg Glu Glu Asp Phe Thr Ser Lys Arg
435 440 445

Ile Ala Thr Gly Phe Ile Ala Asp His Pro His Leu Leu Gln Ala Pro
450 455 460

Pro Ala Asp Asp Glu Gln Gly Arg Ile Leu Asp Tyr Leu Ala Asp Val
465 470 475 480

Thr Val Asn Lys Pro His Gly Val Arg Pro Lys Asp Val Ala Ala Pro

485 490 495

Ile Asp Lys Leu Pro Asn Ile Lys Asp Leu Pro Leu Pro Arg Gly Ser
500 505 510

Arg Asp Arg Leu Lys Gln Leu Gly Pro Ala Ala Phe Ala Arg Asp Leu
515 520 525

Arg Glu Gln Asp Ala Leu Ala Val Thr Asp Thr Thr Phe Arg Asp Ala
530 535 540

His Gln Ser Leu Leu Ala Thr Arg Val Arg Ser Phe Ala Leu Lys Pro
545 550 555 560

Ala Ala Glu Ala Val Ala Lys Lys Thr Pro Glu Leu Leu Ser Val Glu
565 570 575

Ala Trp Gly Gly Ala Thr Tyr Asp Val Ala Met Arg Phe Leu Phe Glu
580 585 590

Asp Pro Trp Asp Arg Leu Asp Glu Leu Arg Glu Ala Met Pro Asn Val
595 600 605

Asn Ile Gln Met Leu Leu Arg Gly Arg Asn Thr Val Gly Tyr Thr Pro
610 615 620

Tyr Pro Asp Ser Val Cys Arg Ala Phe Val Lys Glu Ala Ala Ser Ser
625 630 635 640

Gly Val Asp Ile Phe Arg Ile Phe Asp Ala Leu Asn Asp Val Ser Gln
645 650 655

Met Arg Pro Ala Ile Asp Ala Val Leu Glu Thr Asn Thr Ala Val Ala
660 665 670

Glu Val Ala Met Ala Tyr Ser Gly Asp Leu Ser Asp Pro Asn Glu Lys
675 680 685

Leu Tyr Thr Leu Asp Tyr Tyr Leu Lys Met Ala Glu Glu Ile Val Lys
690 695 700

Ser Gly Ala His Ile Leu Ala Ile Lys Asp Met Ala Gly Leu Leu Arg
705 710 715 720

Pro Ala Ala Val Thr Lys Leu Val Thr Ala Leu Arg Arg Glu Phe Asp
725 730 735

Leu Pro Val His Val His Thr His Asp Thr Ala Gly Gly Gln Leu Ala
740 745 750

Thr Tyr Phe Ala Ala Ala Gln Ala Gly Ala Asp Ala Val Asp Gly Ala
755 760 765

Ser Ala Pro Leu Ser Gly Thr Thr Ser Gln Pro Ser Leu Ser Ala Ile

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770 775 780

Val Ala Ala Phe Ala His Thr Arg Arg Asp Thr Gly Leu Ser Leu Glu
785 790 795 800

Ala Val Ser Asp Leu Glu Pro Tyr Trp Glu Ala Val Arg Gly Leu Tyr
805 810 815

Leu Pro Phe Glu Ser Gly Thr Pro Gly Pro Thr Gly Arg Val Tyr Arg
820 825 830

His Glu Ile Pro Gly Gly Gln Leu Ser Asn Leu Arg Ala Gln Ala Thr
835 840 845

Ala Leu Gly Leu Ala Asp Arg Phe Glu Leu Ile Glu Asp Asn Tyr Ala
850 855 860

Ala Val Asn Glu Met Leu Gly Arg Pro Thr Lys Val Thr Pro Ser Ser
865 870 875 880

Lys Val Val Gly Asp Leu Ala Leu His Leu Val Gly Ala Gly Val Asp
885 890 895

Pro Ala Asp Phe Ala Ala Asp Pro Gln Lys Tyr Asp Ile Pro Asp Ser
900 905 910

Val Ile Ala Phe Leu Arg Gly Glu Leu Gly Asn Pro Pro Gly Gly Trp
915 920 925

Pro Glu Pro Leu Arg Thr Arg Ala Leu Glu Gly Arg Ser Glu Gly Lys
930 935 940

Ala Pro Leu Thr Glu Val Pro Glu Glu Glu Gln Ala His Leu Asp Ala
945 950 955 960

Asp Asp Ser Lys Glu Arg Arg Asn Ser Leu Asn Arg Leu Leu Phe Pro
965 970 975

Lys Pro Thr Glu Glu Phe Leu Glu His Arg Arg Arg Phe Gly Asn Thr
980 985 990

Ser Ala Leu Asp Asp Arg Glu Phe Phe Tyr Gly Leu Val Glu Gly Arg
995 1000 1005

Glu Thr Leu Ile Arg Leu Pro Asp Val Arg Thr Pro Leu Leu Val Arg
1010 1015 1020

Leu Asp Ala Ile Ser Glu Pro Asp Asp Lys Gly Met Arg Asn Val Val
1025 1030 1035 1040

Ala Asn Val Asn Gly Gln Ile Arg Pro Met Arg Val Arg Asp Arg Ser
1045 1050 1055

Val Glu Ser Val Thr Ala Thr Ala Glu Lys Ala Asp Ser Ser Asn Lys

1060 1065 1070

Gly His Val Ala Ala Pro Phe Ala Gly Val Val Thr Val Thr Val Ala
1075 1080 1085

Glu Gly Asp Glu Val Lys Ala Gly Asp Ala Val Ala Ile Ile Glu Ala
1090 1095 1100

Met Lys Met Glu Ala Thr Ile Thr Ala Ser Val Asp Gly Lys Ile Asp
1105 1110 1115 1120

Arg Val Val Val Pro Ala Ala Thr Lys Val Glu Gly Gly Asp Leu Ile
1125 1130 1135

Val Val Val Ser
1140

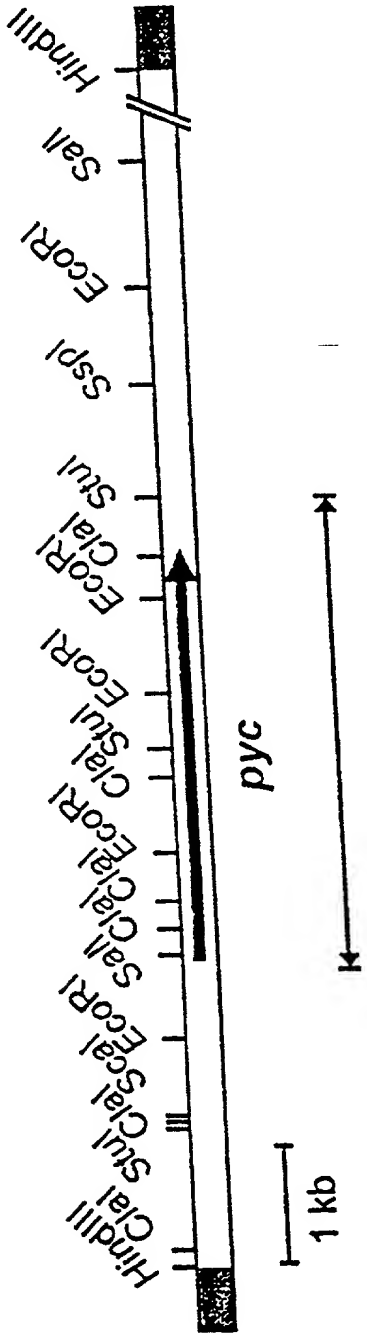
HindIII
SalI
EcoRI
SspI
StuI
ClaI
EcoRI
ClaI
StuI
EcoRI
ClaI
EcoRI
SalI
ClaI
EcoRI
HindIII
ClaI
StuI
ClaI
SspI
EcoRI
pyc
1 kb

FIG. 1

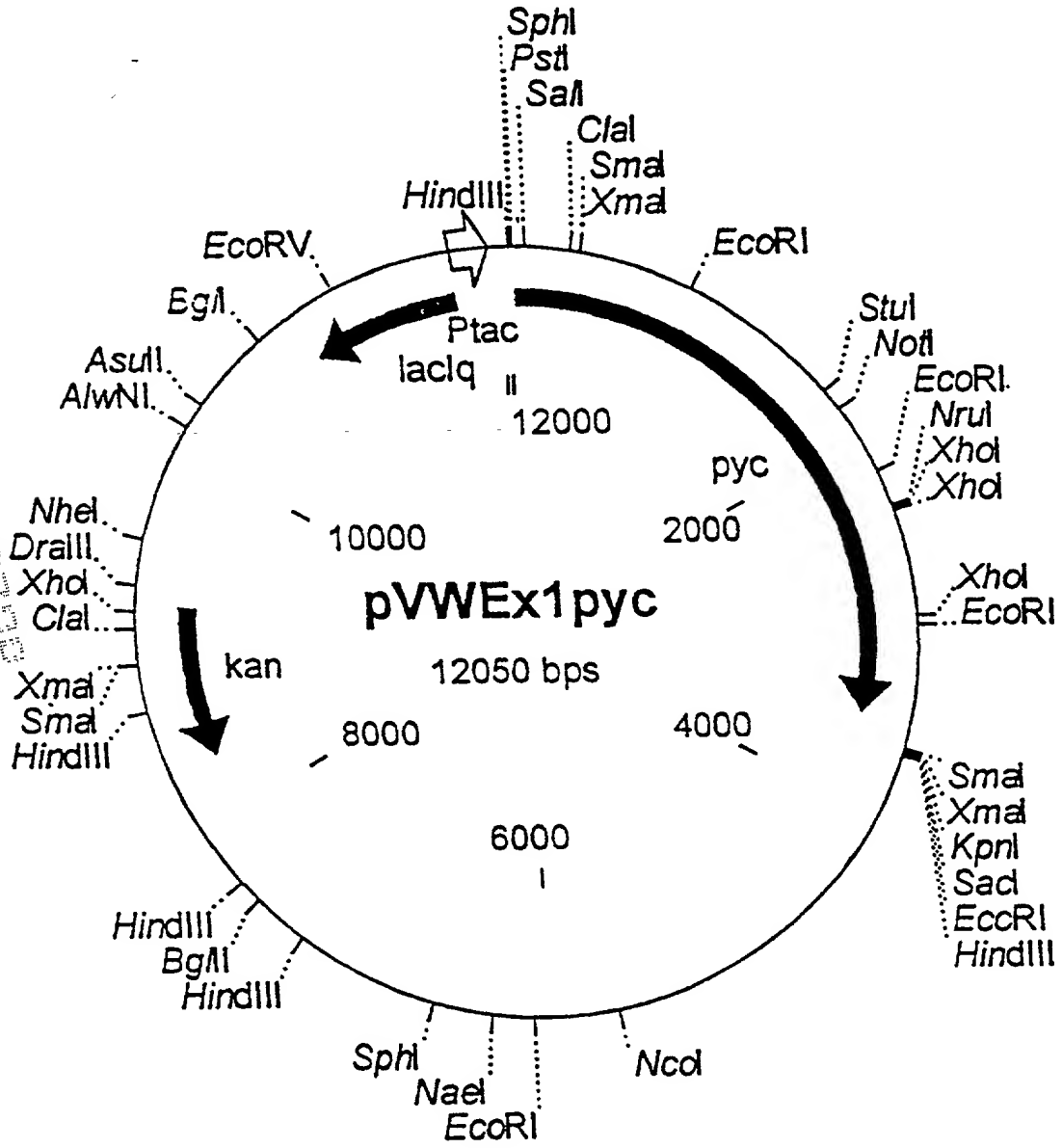
SphI
PstI
SalI
ClaI
SmaI
XmaI
HindIII
EcoRV
EcoRI
EgII
StuI
NotI
Ptac
laclq
AsuII
AlwNI
EcoRI
NruI
XhoI
XhoI
12000
pyc
2000
10000
NheI
DraIII
XhoI
ClaI
pVWEx1pyc
XhoI
EcoRI
kan
12050 bps
XmaI
SmaI
HindIII
8000
4000
SmaI
XmaI
KpnI
SacI
EccRI
HindIII
6000
HindIII
BglI
HindIII
SphI
NaeI
EcoRI
NcoI

FIG. 2

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DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post-office address, and citizenship are as stated below next to my name,
I believe that I am an original joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE SPARTATE AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METHOD

the specification of which was filed on **30 September 1998** as PCT application **PCT/EP98/06210.**
I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.
I hereby claim foreign priority benefits under 35 USC 119 of any foreign applications for patent or inventor's certificate listed below and have also identified below any foreign applications for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Applications

Country	Number	Filing Date	Priority claimed
DE	**19743894.6**	**4 October 1997**	**Yes**
DE	**19831609.7**	**14 July 1998**	**Yes**

I hereby claim the benefit under 35 USC 120 of the United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application(s) in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose material information as defined in 37 CFR 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Filing Date	Status
PCT/EP98/06210	**30 September 1998**	**Pending**

I hereby appoint as attorneys to prosecute this application and to transact all business connected therewith: **Herbert Dubno**, Reg. 19,752; **Jonathan Myers**, Reg. 26,963; **Andrew Wilford**, Reg. 26,597 and each of them individually.
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Direct all telephone calls to: (718) 884-6600

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or

both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature Date: 20. März 2000

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